Food Chemistry 124 (2011) 1643–1646

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

A simple and small-scale sample preparation technique to determine canthaxanthin in hen egg yolk

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ARTICLE INFO

Article history:
Received 8 July 2009
Received in revised form 2 August 2010
Accepted 9 August 2010

Keywords:
Canthaxanthin
Hen egg yolk
Solid-phase extraction mini-cartridge
HPLC

ABSTRACT

This paper describes an easy, small-scale method of sample preparation followed by a reversed-phase (RP) high-performance liquid chromatography (HPLC) for quantifying canthaxanthin (CX) in egg yolk. The egg yolk extract including CX was purified by using a normal-phase InterSep®-CN mini-syringe-column as a solid-phase extraction column. HPLC was performed on a C8 column with an isocratic mobile phase and diode array detector. The proposed method was validated by the analyses of spiked egg yolk samples; resulting recoveries ($\geqslant$94.5%; RSD $\leqslant$ 2.8%), analytical total time (<1 h/sample), and limit of quantitation (0.5 μg/g). The decision limit and detection capability were 25.04 and 25.07 μg/g, respectively.

1. Introduction

Canthaxanthin (CX) (Fig. 1) is a colouring agent, a xanthophyll, that is naturally found in a variety of plants and animals. It is currently used as a feed additive to pigment the eggs or meat of laying hens, broilers, salmon, and trout. Although the European Union (EU) permits several xanthophylls to be added to the feed of laying hens, the use of chemically synthesised CX is nearly the norm in the UK, France, Germany, Italy, and Spain.

Eggs are a very important and basic food because they are highly nutritious, cheap, and readily available. CX is used to brighten the yolks of chicken eggs in response to consumer demands. Since consumers associate bright product colouration with health and quality, CX is particularly important in the poultry-farming industry as it pigments egg yolks.

CX has been reported to cause liver injury and an eye disorder called CX retinopathy, which is the formation of yellow deposits on the retina (FDA, 2003). Following scientific assessments establishing a link between high CX intake and eyesight problems in humans (due to an accumulation of pigment in the retina), the European Commission adopted a directive in 2003 to reduce the authorised level of CX in animal feed (EC, 2002). The European Food Safety Authority (EFSA) (EFSA, 2007) and the Japanese Ministry of Health, Labour and Welfare (JFCRF, 2009) have set maximum residue limits (MRLs) for the CX in several xanthophylls in egg yolk at 30 and 25 μg/g, respectively, to ensure the safety and appropriateness of eggs for human consumption. Monitoring the presence of CX in eggs is, therefore, an important means of guaranteeing food safety.

In response to the recent expansion in the internal food trade, the development of international standardized methods to determine chemical residues in foods is essential in order to guarantee equitable international trade in these foods and ensure food safety for consumers. For both industrial nations and developing countries, an internal harmonised method for residue monitoring in foods is needed.

The optimal method for the routine monitoring of chemicals such as CX in foods of animal origin must be simple, small scale, economical in time and cost, and must cause negligible harm to the environment and analysts. Reducing the use of organic solvents and reagents is an important goal in terms of environmental conservation, human health, and the economy. Although techniques based on high-performance liquid chromatography (HPLC) for the identification of xanthophylls such as CX in poultry egg yolks have been reported (Abdollahi, Rosenholtz, & Garwin, 1993; Bononi, Commissati, Lubian, Fossati, & Tateo, 2002; Bortolotti, Negro, Surai, & Prieto, 2003; Hamilton, 1992; Majchrzak & Elmadfa, 1997; Schlatterer & Breithaupt, 2006), no optimal method that satisfies the aforementioned requirements has yet been identified. Residue analysis of egg is difficult because the formation of an emulsion hinders the recovery of residues and coextracts interfere, both of which require multiple clean-up steps.

This article describes an easy and small-scale technique to strictly monitor CX residue in egg yolk with minimised organic solvent consumption.

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doi:10.1016/j.foodchem.2010.08.017

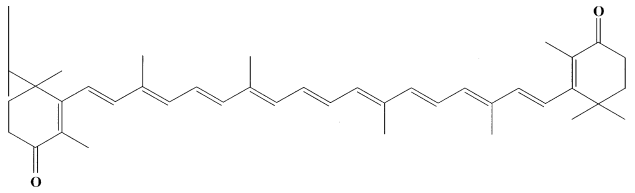

Fig. 1. Chemical structure of canthaxanthin (CX).

2. Experimental

2.1. Materials

All chemicals, organic solvents (acetone, acetonitrile, and *n*-hexane), distilled water (DW), and canthaxanthin (CX) (β,β-carotene-4,4′-dione, ⩾98% purity) standard were purchased from Wako Pure Chem. Ltd. (Osaka, Japan). The organic solvents and DW were HPLC grade. A stock CX standard solution of 100 μg/ml in acetone was taken into cryo-vial, sealed and stored at −80 °C, and protected from light until further use. Working standard solutions were prepared by diluting the stock solution with acetone.

The following apparatuses were used in the sample preparation: a handheld ultrasonic-homogenizer (model HOM-100, 2 mm I.D. probe, Iwaki Glass Co., Ltd., Funabashi, Japan); a microcentrifuge (Biofuge® fresco, Kendo Lab. Products, Hanau, Germany); a vacuum rotary evaporator (model EYELA N-1 M, Tokyo Rikakikai Co., Ltd., Tokyo, Japan); a 0.45 μm disposable syringe filter unit equipped with hydrophilic cellulose acetate membrane (Advantec, Toyo Roshi Co., Ltd., Tokyo, Japan); four normal-phase silica-based sorbent types of InertSep® solid-phase extraction (SPE) mini-syringe-column (100 mg sorbent mass/1 ml reservoir volume), InertSep CN (cyanopropyl), -2OH (glycerylpropylsilylation silica gel), -SI (silica gel), and -FL (florisil) (GL Sciences, Inc., Tokyo, Japan). These InertSep mini-columns were generous gifts from Mr. Yoshimura (Osaka 2nd Sales Dept., GL Sciences).

2.2. HPLC

The HPLC system included a model PU-980 pump and DG-980–50 degasser (both from Jasco Corp., Tokyo, Japan), as well as a model SPD-M10A VP diode array detector (DAD) (Shimadzu Scientific Instruments, Kyoto, Japan). The analytical column was a LiChrospher® 60 RP-Select B (particles of silica with Octyl derivative, C8) (125 × 4 mm, 5 μm d_p, 360 m^2/g surface area, 60 Å pore diameter, 0.9 ml/g pore volume, 11.5% carbon content) column (Kanto Chemical Co., Inc., Tokyo) equipped with a guard column (4 × 4 mm) containing the same packing material using an acetonitrile–water (9:1, v/v) as the isocratic mobile phase at a flow rate of 1.0 ml/min at 45 °C. DAD was operated at 190–600 nm: monitoring wavelength for the CX was adjusted to 470 nm which is an absorption maximum for the analyte. The injection volume was 20 μl.

2.3. Egg yolk samples

To obtain egg yolk samples for the present study, total 10 laying hens (White Leghorn, aged 30 weeks) that were kept in individual cages were used. Feed and water were given *ad libitum*. Eggs from five laying hens that were given CX-free basal layer diet continuously were collected and used as blank eggs. In order to validate the present method for routine monitoring, eggs with residual CX from other five laying hens that fed a diet containing 5 mg/kg CX for 3 weeks were also collected and used as real eggs. These egg yolks that were separated from their albumen were uniformed fully and used as blank and real egg yolk samples, respectively, and stored at −80 °C until analysis.

2.4. Sample preparation

An accurate 0.1 g sample was taken into a 1.5 ml micro-centrifuge tube and homogenised with 0.6 ml of acetone with a handheld ultrasonic-homogenizer for 30 s. After being homogenised, the capped tube was centrifuged at 12,000*g* for 5 min. The supernatant was evaporated to dryness. The residue was dissolved in 0.5 ml of hexane and poured to the InertSep CN mini-column. After the column had been washed with 1 ml of hexane, CX was eluted with 1 ml of acetone–hexane (2:8, v/v). The eluate was evaporated to dryness and the residue was dissolved in 1 ml of the HPLC mobile phase. The solution was filtered through the 0.45 μm filter unit and the filtrate was injected into the HPLC system.

3. Results and discussion

3.1. Assay conditions

As carotenoids, including CX, are light-labile, a stock CX standard solution was put into a cryovial, sealed and stored at −80 °C, and protected from light until further use. Working CX standard solutions were freshly prepared on the day of analysis, and assays were performed under a darkened room condition to protect against light exposure.

3.2. Sample preparation

The method used in this study is an easy and small-scale technique that minimises organic solvent consumption in the preparation of CX in egg yolk samples. In the extracting operation, the resulting extract did not form an emulsion that would hinder CX recovery. The obtained extract was further cleaned using normal-phase SPE mini-columns.

Four InterSep mini-columns packing normal-phase materials were used as normal-phase SPE columns. The retention profiles of CX in these columns were compared. The possibility of eluting CX from the columns using several solvents was evaluated. Since the normal-phase sorbents were de-retained with the addition of acetone to the eluent, the effect of the concentration of acetone in the eluent (acetone–hexane, v/v) on the recovery of CX standard from the InterSep columns was determined when the elution volume was standardised at 1 ml. The resulting profiles are presented in Table 1. The eluent that best recovered CX (>95%) from the three columns was acetone–hexane (2:8, v/v). Because CX was not eluted with hexane from InterSep-SI, -FL, and -CN (except for -2OH) columns, hexane was used as the washing eluent to eliminate interfering compounds of sample origin. Prewashing of the SPE column using hexane as a prior eluate was included in the

Table 1
Effect of the acetone concentration in the eluent (acetone–hexane) on the recoveries of CX from InertSep® SPE mini-syringe-columns.

Packing sorbent[a]	Acetone–Hexane (v/v)			
	0:10	1:9	2:8	3:7
SI	0	90	98	–[b]
FL	0	76	99	–
CN	0	85	98	–
2OH	6	85	96	–

Data are average recoveries (%, $n = 3$). A standard solution containing 25 μg of CX was applied to the column. The volume of the eluent was standardised at 1 ml.

[a] See Section 2 in text.

[b] Not tried.